CATHELCO LTD
BALLAST WATER MANAGEMENT SYSTEM

PART 4 ANNEX 1b

NIOZ QAPP and Test Protocol

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Quality Assurance Project Plan (QAPP) for the Biological Efficacy Testing of the Cathelco Ballast Water Treatment System from Cathelco GmbH as part of the Type Approval Process under Resolution MEPC.174(58)

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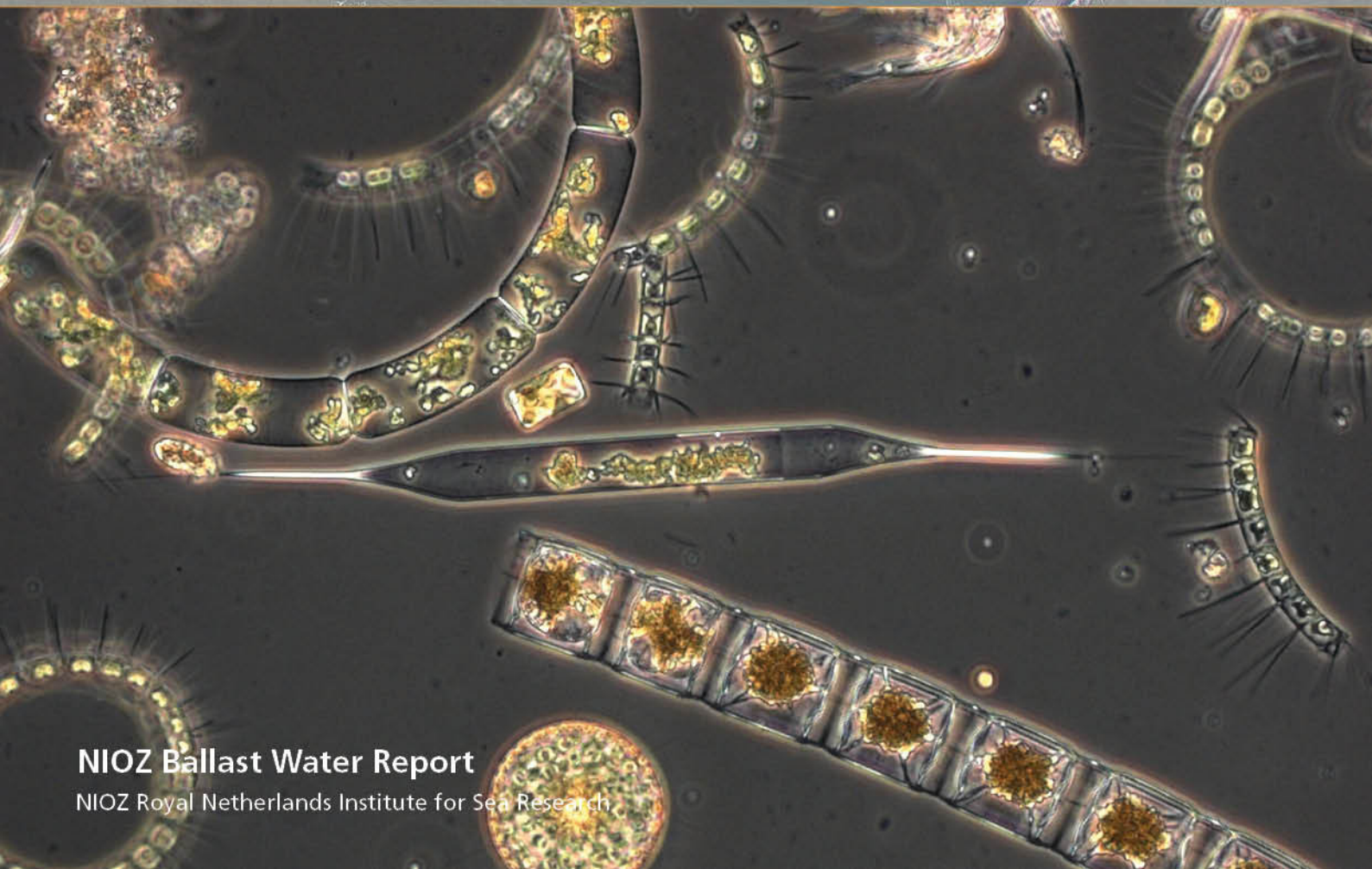
L. Peperzak



NIOZ Ballast Water Report
NIOZ Royal Netherlands Institute for Sea Research

QUALITY ASSURANCE PROJECT PLAN (QAPP) FOR THE BIOLOGICAL EFFICACY TESTING OF THE CATHELCO BALLAST WATER TREATMENT SYSTEM FROM CATHELCO GMBH AS PART OF THE TYPE APPROVAL PROCESS UNDER RESOLUTION MEPC.174(58)

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September 2012 (version 2)

Signed on Texel, The Netherlands on: 10 September 2013

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ABSTRACT

This Quality Assurance Project Plan (QAPP) is the project-specific technical document reflecting the specifics of the test facility, the BWTS tested, and other conditions affecting the actual design and implementation of the required experiments. The QAPP consists of general information, information on the NIOZ test facility and the Cathelco BWTS, test water quality, sampling and sample storing, the measurement of variables and QA/QC.

In September 2012 this QAPP was sent to the BSH as section 2 of the "Project Plan (QAPP) for the Biological Efficacy Testing of the Cathelco Ballast Water Treatment System from Cathelco GmbH as part of the Type Approval Process under Resolution MEPC.174(58)" (version 2). In August 2013, on request by the BSH, the text was formatted as a stand-alone document. This stand-alone document includes the correct process diagram of the Cathelco BWTS.

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1 INTRODUCTION

The QAPP detailed in this document forms the basis for the biological efficacy testing of the CATHELCO Ballast Water Treatment System (BWTS) manufactured by Cathelco GmbH (Kiel, Germany) in 2012 at the ballast water treatment facility of the Royal Netherlands Institute for Sea Research in The Netherlands. The first version of this protocol was submitted to the BSH (Bundesamt für Seeschifffahrt und Hydrographie or Federal Maritime and Hydrographical Agency, Hamburg, Germany) in March 2012 in advance of the land-based tests that started on April 12 of that year.

The QAPP is a project specific technical document reflecting the specifics of the BWTS tested, the test facility, and other conditions affecting the actual design and implementation of the required experiments. On the other hand, the QMP should address the quality control management structure and policies of the test facility. In 2012 the combined QAPP and the QMP, collectively known as the quality control/quality assurance (QA/QC) program, was extensively extended when compared to previous years.

Nevertheless, the BSH invited dr. A. Cangelosi (Northeast-Midwest Institute, USA) to review the QA/QC section of the protocol. In addition, NIOZ commissioned a review of the entire protocol to dr. S. Kools (Grontmij, The Netherlands). The present version of the protocol is revised in reaction to these two reviews.

In the summer of 2012 the BSH decided that a sufficient number of marine (saline) tests had been performed earlier that year and that five successful freshwater tests were needed for type approval. This meant that additional information had to be added on freshwater sampling and testing in the relevant chapters of this protocol.

The tests outlined in this protocol will evaluate the biological efficacy as outlined in the Guidelines for Approval of Ballast Water Management Systems, G8, adopted on 22 July 2005 as Resolution MEPC.125(53) (IMO, 2005) and as adopted in revised form on 10 October 2008 as Resolution MEPC.174(58/23, Annex 4) of the Ballast Water Convention of the IMO (IMO 2008).

The test protocol will be submitted for Type Approval by the applicant (Cathelco GmbH) to the German National Administration (BSH).

The Cathelco BWTS is composed of two main modules to disinfect ballast water of ships. One module is a filtration system, the second a UV-reactor. Both are operated during ballast water intake. During de-ballast operations the ballast water from the tanks passes again through the UV-reactor before discharge.

The applicant gives a brief description of the main components of the BWT system in chapter 2.

A detailed description of the test facility and the design of the test, sampling, sample storage and descriptions of the measurements of abiotic as well as biological variables are given in ensuing chapters. Because NIOZ is an academic research institute additional methods to count organisms and to establish their viability are continuously being developed. These methods may be applicable to efficacy testing according to the present as to potentially future D-2 Ballast Water Performance Standards and G8-guidelines.

2 GENERAL DESCRIPTIONS

Figure 1. Aerial view of the NIOZ harbour (lower right), NIOZ and the TESO ferry connecting the island of Texel with the main land (top). The Mokbaai is the source for additional suspended solids. ©Photo: Simon Smit Photography, Den Burg, Texel.

2.1 NIOZ profile

All tests of the Cathelco BWTS will be carried out under supervision of the Royal Netherlands Institute for Sea Research, Landsdiep 4, 1797 SZ 't Horntje, Texel, The Netherlands (from here on NIOZ, for details see: www.nioz.nl).

NIOZ Royal Netherlands Institute for Sea Research is the National Oceanographic Institute of the Netherlands. NIOZ is an institute of the Netherlands Organization for Scientific Research (NWO). The institute employs about 340 people at locations on the island of Texel on the border of the North Sea and the Wadden Sea (main location) and in Yerseke in the southwest of the country. The annual budget is approximately €30 million.

The mission of NIOZ is to gain and communicate scientific knowledge on coastal seas and oceans for a better understanding of the system and sustainability of our planet, to manage the national facilities for sea research and to support research and education in the Netherlands and in Europe.

In order to fulfil its mission, the institute performs tasks in four specific fields.

Research: The emphasis is on innovative and independent fundamental research in continental seas and open oceans. Increasingly, the institute also carries out research based on societal issues. The senior scientists at NIOZ all participate in international research projects. Several of them also hold a professorship at Dutch or foreign Universities.

Education: The institute educates PhD students and master students of universities and schools for professional education. Together with several universities, NIOZ also organises courses for PhD students and master students in the marine sciences. A number of our senior scientists is also appointed as professor at Dutch and foreign universities.

Marine Technology: NIOZ has its own workshops for mechanical, instrumental en electronic engineering. Here, marine research equipment is being designed and built according to the wishes of our individual scientists.

Facilities: NIOZ invites marine scientists from Dutch and foreign institutes and universities to write scientific proposals involving the institute's research vessels, laboratories and large research equipment. Our ocean-going research vessel 'Pelagia' is shared on a European level in the 'Ocean Facilities Exchange Group' (www.ofeg.org)

The basic scientific disciplines at NIOZ are physics, chemistry, biology and geology. Multidisciplinary sea research is regarded as one of the main strengths of the institute. Therefore, the research is organised in 5 multi-disciplinary themes: 'Open ocean processes, Sea floor dynamics, Wadden and shelf sea systems, Climate variability and the sea and Biodiversity and ecosystem functioning'.

Together with a number of oceanographic partners, NIOZ also maintains the popular marine website www.seaonscreen.org.

For more information, please contact our Communication & PR department at cpr@nioz.nl, or visit our website at www.nioz.nl
NIOZ has extensive experiences in the field of ballast water and ballast water treatment technologies at its harbour on the island of Texel. During the past seven years several pilot tests for ballast water treatment were conducted in the NIOZ harbour and so far, between 2007 and 2010 seven full scale land-based tests were carried out for Final and Type Approval.

2.2 Profile of the Cathelco Group

Cathelco Ltd was formed in 1956 and has become a world leading supplier of cathodic protection equipment to the shipping and offshore marine markets.

The parent company based in Chesterfield, United Kingdom manufactures marine pipe work anti-fouling (AF) and impressed current cathodic protection (ICCP) systems.

The Cathelco Group has grown through a series of acquisitions beginning with Corrintec Ltd in 1995. Today, Corrintec Marine continues to operate as a wholly owned subsidiary serving the military sector worldwide.

In 2005, the company established Cathelco Korea, a subsidiary engaged in manufacturing and distribution serving the Korean shipbuilding market.

More recently, in 2010, Seafresh Desalinators Ltd was acquired which specialises in reverse osmosis water makers from a manufacturing facility in Bournemouth, United Kingdom.

To serve the shipbuilding and repair industry in South East Asia, Cathelco S.E.A. based in Singapore, became a subsidiary in 2011.

In addition to its marine engineering activities, the Cathelco Group encompasses Casting Repairs Ltd, specialising in the repair of architectural cast ironwork. It also has an active property division, focusing on property development and rental via the Broomco Ltd subsidiary.

Cathelco GmbH was established in 2010 in Kiel, Germany, to research and develop ballast water treatment equipment for the worldwide market. The aim is to develop a chemical free, two step-ballast water treatment system.

This system should be easy to retrofit for existing vessels and innovative for new builds.

Beside the ballast water issue, the Cathelco R&D Centre provides also services to the Cathelco Group in respect of testing and improving of existing products. This includes but is not limited to antifouling systems for vessels and other marine structures.

2.3 Technical Overview of Cathelco's BWTS

Cathelco has developed a 100% chemical free BWTS (Figure 2). It is based on the combination of filtration + UV treatment. There are no active substances needed for the treatment of the ballast water, or for the cleaning of the UV system. The system was designed with emphasis on retrofitting (e.g. installation of the different components as required by different engine room designs). However, skid-mounted systems will also be available, which are optimized for foot-print.

Cathelco's BWTS is a modular ballast water management system. The system is installed in bypass to the main ballast line and provides a safe, flexible and economical process for the treatment of ballast water and eradication of aquatic invasive species. Treatment of ballast water is achieved through a simple and efficient two-step process.

1. Filtration

The ballast water passes through an automatic back flushing filter capable of removing particulates, and organisms (zooplankton and phytoplankton) using a 40 µm super mesh screen. The screen is arranged in cone shaped filter candles, where the number of filter candles varies with the treatment related capacity (TRC) of the BWTS. During the NIOZ G8-tests to be performed at 200 m^3/h the number of filter candles will be nine.

The automatic cleaning cycle of the filter is activated by an increased pressure drop across the filter. The filter candles are cleaned one after the other without interruption of the filtration process. The frequency of the filter back flushes depends on the quality of the water (e.g. the TSS content). The concentrate is discharged over board. This ensures that the screen is kept clean and the filtration process maintained at maximum efficiency at all times.

2. UV disinfection

The filtered ballast water is directed into the disinfection chamber where a cross flow arrangement with two medium pressure ultraviolet lamps delivers the high intensity irradiation. The UV light intensity is continuously monitored during system operation so that intensity is maintained above pre-set values to ensure delivery of the required dose. The ultraviolet lamps are housed within quartz sleeves and an automatic mechanical cleaning system minimizes bio-fouling and controls the accumulation of deposits on the UV lamp sleeves.

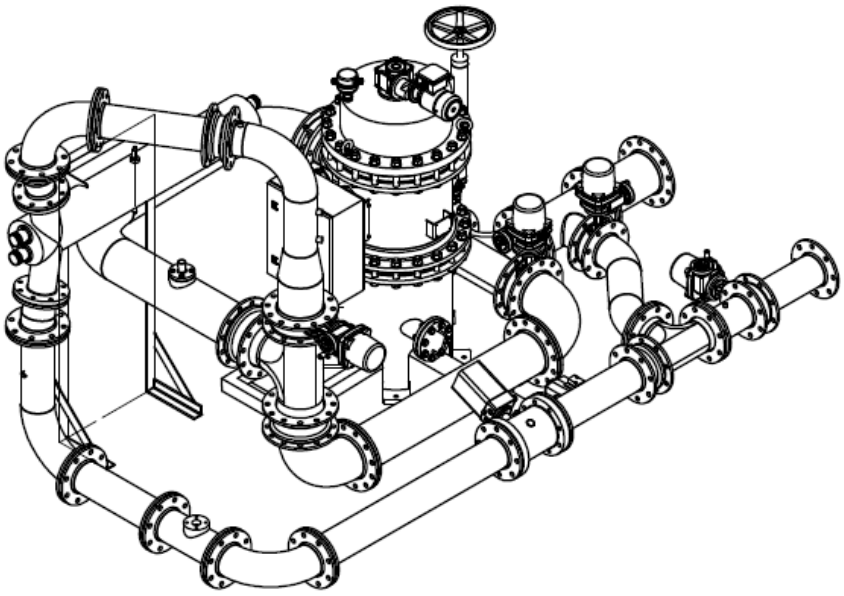

Figure 2. The Cathelco ballast water treatment system to be tested at NIOZ in 2012. This design allows for an easy exchange of the filters.

At discharge, the automatic backwash filter is by-passed and the ballast water is pumped from the tanks through the UV disinfection chamber only. Thus the ballast water is subjected to UV disinfection treatment prior to discharge overboard.

Specific features/ advantages of Cathelco's BWTS

The filtration step

Cathelco's BWTS can be operated with two different types of filters, giving it the maximum possible flexibility in addressing different installation requirements.

The UV-lamp system

Cathelco's BWTS uses special medium pressure UV lamps with a reduced Mercury content. These lamps are mounted to a solid flange and they are surrounded / protected by a sealed robust quartz sleeve. This complete UV-lamp system, containing two lamps, is fitted to the UV reactor by a few screws. Access to the UV reactor for maintenance is from a single side only.

The lamp recycling scheme

On an annual basis, the UV-lamp system should be send off to Cathelco for refurbishment. The renovated lamp system will be returned to the ship with a new 1 year operating guarantee, if the lamp system has not been installed for a total time of more than 2 years.

The automated cleaning system of the UV

This is the first NON-chemical in place cleaning system for UV-reactors (CIP-system). It uses rubber cleaning elements that are supplied on demand to the UV reactor after the BW operation is finished. The cleaning process is triggered by the intensity measurement of the individual UV lamps. Start, stop and duration of the cleaning process are controlled by Cathelco's BW system. No manual interference is needed.

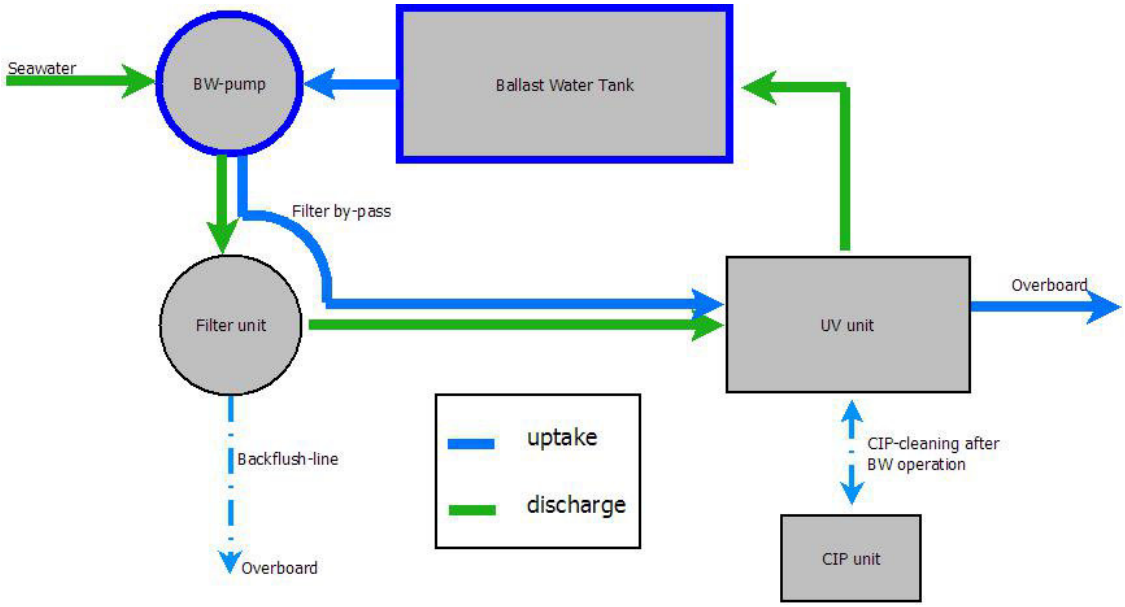

Figure 3. Process diagram of Cathelco's BWTS.

Calculation of the UV dose

The UV dose is calculated directly from the water quality (UV transmittance of the water) and from the flow rate. The use of single electronic ballast units for each individual UV lamp allows for a maximum of flexibility in the power consumption. The UV lamps are operated at the most suitable dose rate at any time, allowing for significant savings in overall power consumption of the system.

The UV dose at T10=70% will be about 135 J/m² at 200m³/h flow rate. This dose is above the requirements for a 4 log reduction of many microorganisms, if no photo repair mechanism occurs.

As there is no light inside the ballast water tanks, our system accepts this dose during ballasting only. If the UV-T decreases further, the flow will be reduced automatically to ensure that the minimum dose (133 J/m²) is maintained.
During de-ballasting, the water is much clearer, i.e. UV-T is higher, and the Cathelco system will dose between 400 J/m² and 700 J/m², which is well above the dose requirements (for a 4 log reduction) for the majority of organisms, even in the presence of photo repair mechanisms.

Cathelco's BWTS will be tested at a treatment related capacity (TRC) of 200 m³/h during the land based Type Approval Tests at the NIOZ test facility. There will be two UV-reactors and two different filters involved during the tests. The general layout of the test system should be according to the P&ID in Figure 4.

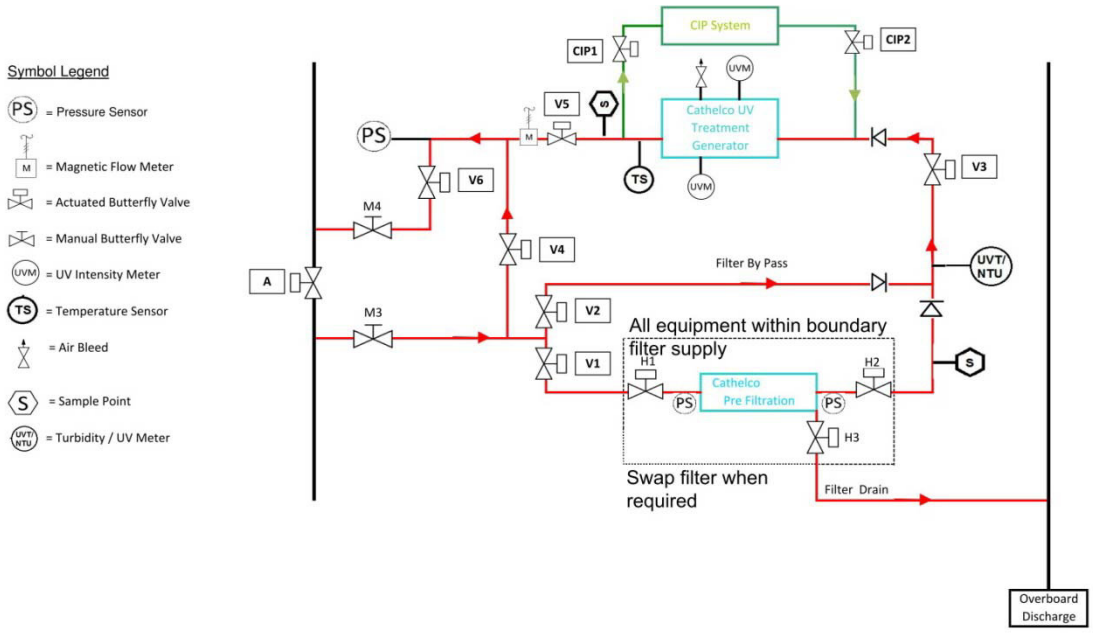

Figure 4. Piping and Instrumentation diagram of Cathelco's BWTS for a TRC (treatment related capacity) of 200 m3/h.

3 TEST FACILITY

3.1 NIOZ test facility

The land-based tests will be carried out on the island of Texel (NIOZ harbour, NL) from March to July (spring and early summer season). The NIOZ test-site is equipped with two times (Navicula and Pelagia quay) three silos or underground storage tanks of 300 m³ each to simulate ship's ballast water tanks (Figure 5).

The NIOZ harbour is located at the Marsdiep tidal inlet between the North Sea and the Wadden Sea. By sampling in different phases of the tidal cycle, waters from different seas with different abiotic and biological characteristics can be used in G8-tests. The area as a whole is characterized by a rich and varying biodiversity and high number of various planktonic organisms, especially in the spring and early summer period. During the test cycles the numbers of organisms present in the water will continuously be monitored to assure that the validity of the test cycles is in accordance with § 2.3.20 of the G8-guidelines.

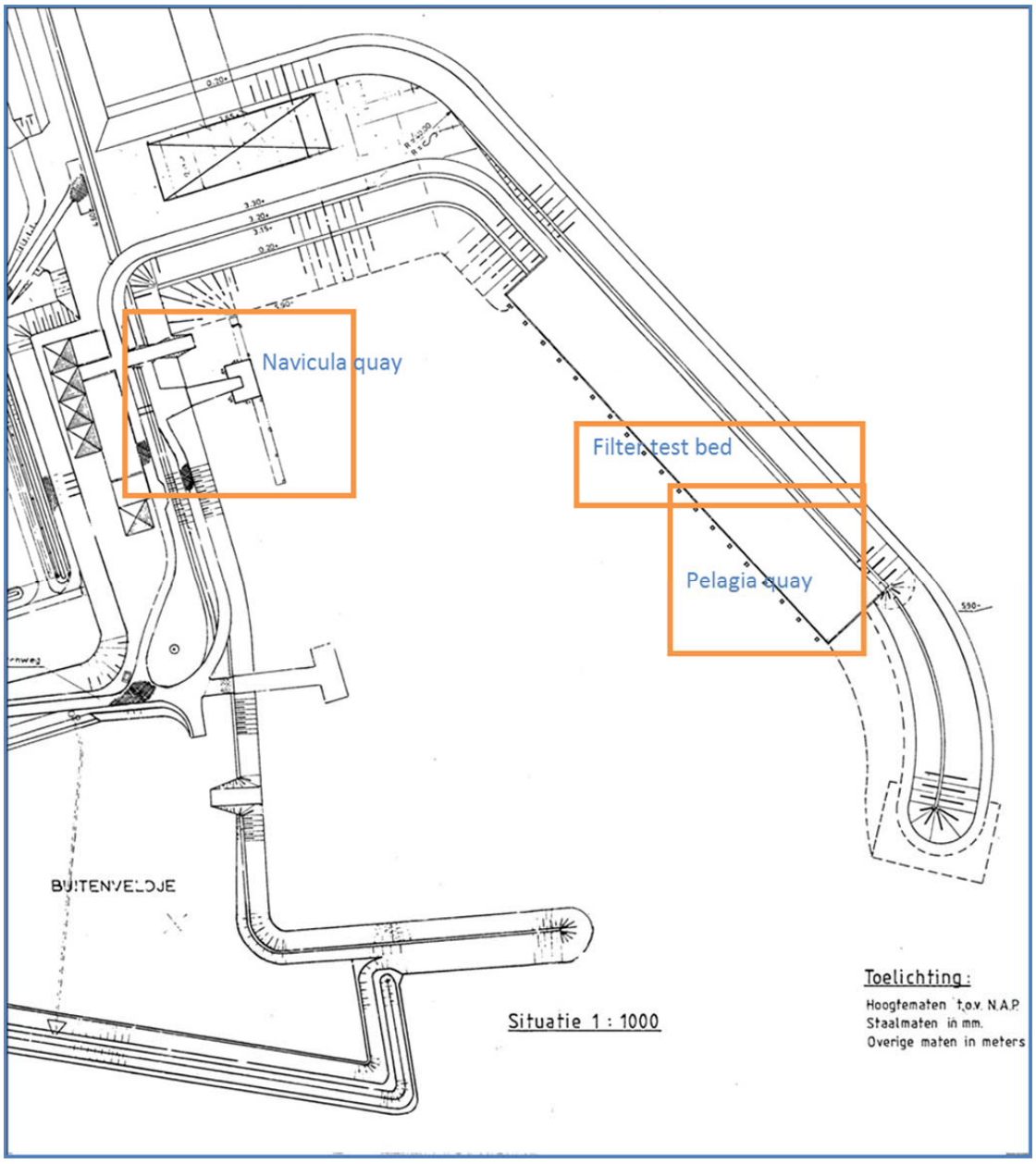

Figure 5. Schematic of the NIOZ harbour test facilities, including the two quays (Navicula and Pelagia) that are used in G8-tests.

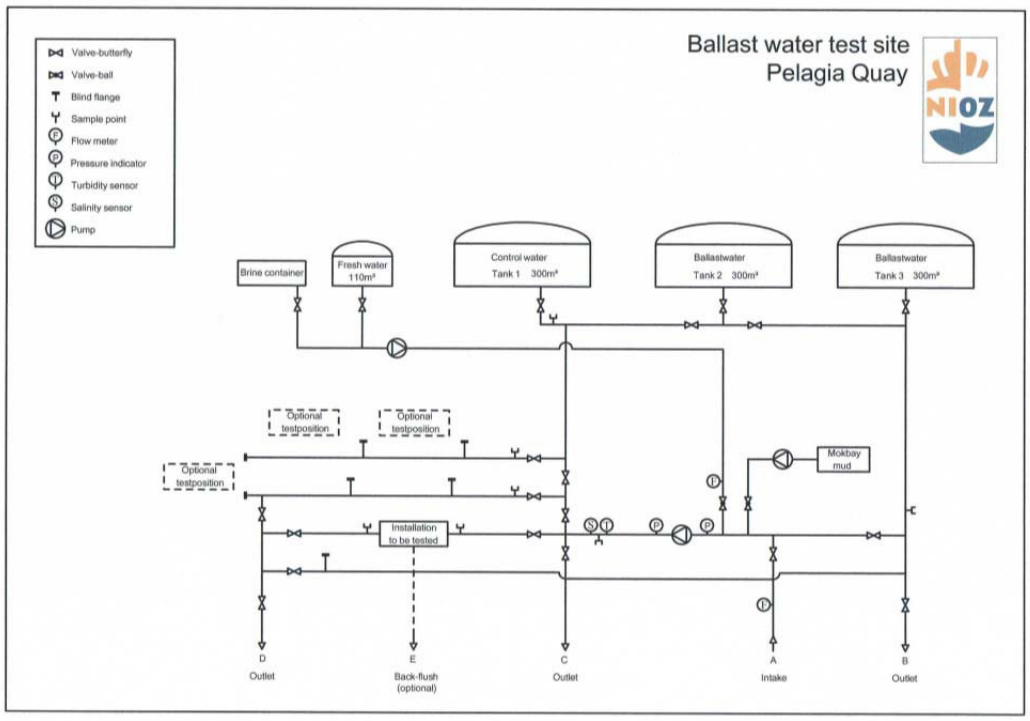

Figure 6. Piping and Instrumentation diagram of the Pelagia quay test site at the NIOZ harbour. The installation to be tested is a UV-treatment system. The installation consists of three ballast water tanks, one for control (untreated) water and two for treated water. Brine or fresh water can be added to adjust the salinity of the test water. Mud can be added to increase the concentration of Total Suspended Solids (TSS). Flow rates, system pressures, salinity and turbidity are monitored during intake and discharge. P&I diagrams are available for both Navicula and Pelagia quays and for different intake and discharge scenarios.

3.2 Freshwater intake in Den Helder and transport to NIOZ

Fresh water in Den Helder will be taken in at location B (Figure 7) by a ship that normally transports gravel and sand with a capacity of 650 m^3. The ship will be cleaned of any remaining sand before taking in water. The intake water will be pumped in the hold with tubes that are suspended at a height of maximally 1 meter below the water surface. Intake will take place on the afternoon prior to the tests.

The Den Helder harbour is monitored by NIOZ partner IMARES. According to IMARES Den Helder harbour is eutrophic and very productive. In May-July 2012 the concentration of >50 µm organisms was between 68,000 and 22,100,000 m^{-3}. For the 10-50 µm organisms this was 397 to 8,040 per mL. IMARES' experience at another location in The Netherlands learned that organism concentrations can remain high up till winter. Only when water temperature drops below ca. 7°C the zooplankton production will stop. Such low temperatures can be expected in November-December, but not during the planned G8-tests in September-October.

The freshwater test water is transported over a relatively short distance across the Marsdiep tidal inlet of the Wadden Sea within 12 hours by ship to the NIOZ harbour (Figure 8). The day following intake at Den Helder, i.e. within 24 hours, the test water is available in the NIOZ harbour and ready to be pumped into the NIOZ harbour installation.

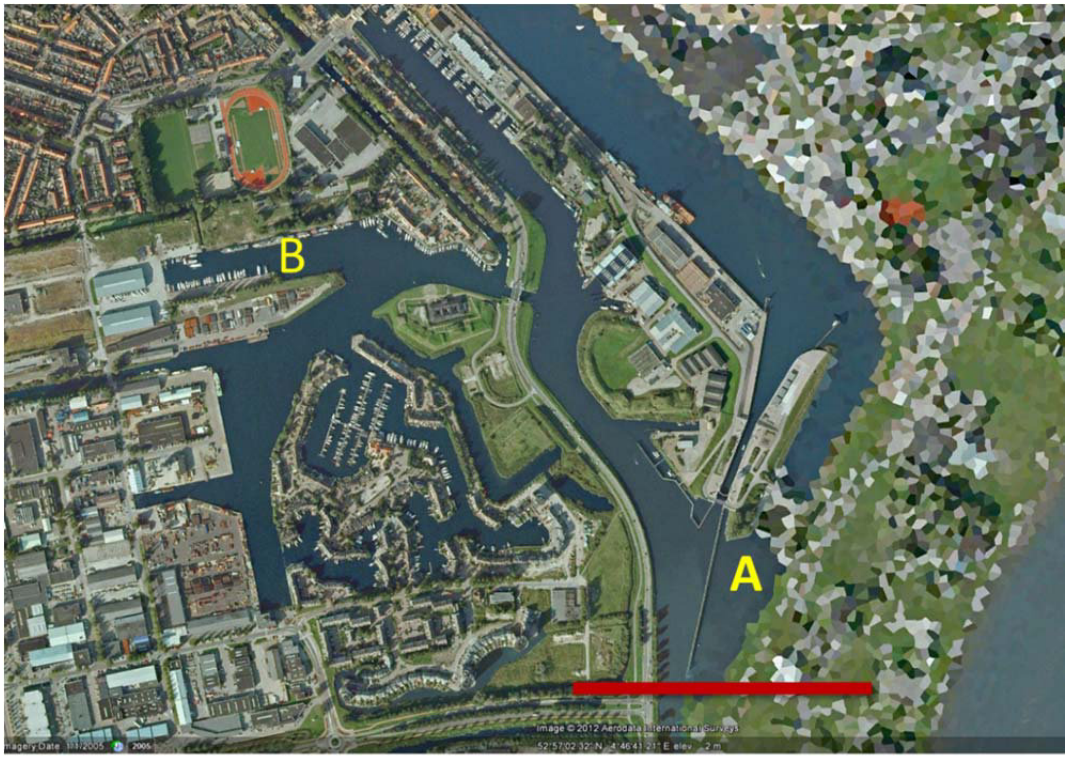

Figure 7. Freshwater intake at B („Spoorhaven"), the IMARES monitoring site in Den Helder harbour. The red horizontal bar is 0.5 km. Site A was the regular monitoring site; site B is the new IMARES monitoring site where fresh water for BWTS testing at NIOZ will be collected.

Figure 8. The test water will be transported to the NIOZ harbour over a distance of 6.5 km in less than 12 hours.

The pump to be used is an Amarex KRTK 100-401/354WG-S that will be suspended by a crane from the NIOZ Pelagia quay (Figure 9). The crane will be able to steadily position the pump because the freshwater surface level will change according to deballasting and tide. This pump will be connected to the NIOZ installation (Figure 6) by a flexible hose.

Figure 9. The Amarex pump to be used in pumping fresh water from a boat into the NIOZ installation.

3.3 BWTS test set-up: treatment and control tanks

A typical test of a treatment system is performed with two treatment tanks and one control tank that are filled in rapid succession, i.e. on the same day at approximately the same time in the tidal cycle (Figure 6). The control tank with untreated water serves as reference to examine the effect of the treatment, including holding for at least 5 days (§2.3.35 G8-guidelines). The control tank can also indicate an unexpected source of mortality due to the testing arrangement (§2.3.37 G8-guidelines). Therefore, the average discharge results in the control water should not be less than or equal to 10 times the values in regulation D-2.1 (§2.3.36 G8-guidelines).

3.4 Toolbox meetings

The general test set-up is described in Figure 6 and in §3.2. Directly prior to each test a tool box meeting is held on the quay site with all team members. During this meeting all actions, such as order of tanks to be filled and flow rates, are briefly discussed.

3.5 Ballast water book

All manufacturers should log their activities in a ballast water book issued by the NIOZ. Several books may be issued during the entire test period. These books remain at NIOZ in the appropriate dossier.

3.6 Test season: time planning

The intermediate and high salinity range test season at the NIOZ harbour is restricted to spring and summer. In this period of the year sufficiently high numbers of organisms are naturally present in the North and Wadden Sea. At NIOZ the test water is not enriched with organisms, neither artificially cultured nor collected at sea. In general, early spring sea water has a lower salinity then in summer due a decrease in river discharge. A decrease in wind speed during spring will lead to diminishing concentrations of total suspended solids (TSS) and, therefore, sediment from the nearby Mokbaai (Figure 1) is added to increase TSS to the required value of 50 mg/l for brackish water.

The freshwater test season, according to IMARES data, begins in March and probably ends in November-December.

In February sampling and measurements in the harbour start in order to monitor the start of the spring plankton bloom. In March the first G8-tests may be performed, depending on natural circumstances such as water temperature and underwater light climate that affect the plankton development.

The first set of tests is carried out at the intermediate salinity range of G8 (§2.3.17) because in early spring the freshwater content of the Wadden Sea is relatively high. Test water is pumped from the harbour at low tide when low salinity Wadden Sea water flows towards the North Sea. The second set of high salinity tests is performed in late spring or early summer. Test water is pumped from the harbour at high tide when relatively saline North Sea water flows towards the Wadden Sea.

After consultation with the BSH, the Cathelco BWTS tests carried out in spring and early summer of 2012 were combined to one salinity (saline) range. This meant that an additional series of freshwater tests had to be performed. The first appropriate months for these freshwater tests were September and October.

4 TEST WATER: QUALITY, SAMPLING, STORING

4.1 Abiotic quality

The land-based test cycles have to be carried out at specific water qualities as defined in the G8-guidelines. The NIOZ-harbour represents a brackish water environment with a varying salinity (20 – 35 PSU). High salinity water originating from the North Sea is taken in around high tide. Low salinity water from the Wadden Sea is taken in around low tide. The salinity of the Wadden Sea water is dependent on the discharge of fresh water from Lake IJssel, which itself depends on the amount of rainfall and on the flow rates in the rivers Rhine and IJssel. In an effort to maintain a minimum 10 PSU salinity difference as requested under § 2.3.17 of G8, per tank 15m^3 fresh water is added in the pipelines to the natural water prior to the pump to reduce the ambient salinity (ca. -2 PSU) and 8 m^3 brine (100 kg m^{-3} industrial quality salt) is added to increase salinity (ca. + 2 PSU) at the second set of test series. At present only brackish and high salinity seawater conditions can be tested at NIOZ.

Figure 10. Extra natural sediment from the "Mokbaai" will be added when required to meet the minimum TSS concentration for the given salinity test cycle.

In addition, per ballast tank 20 litre of mud (15.6 kg dry weight) from the nearby Mokbaai (Figure 1) will be added to the low salinity tests in order to reach the required TSS value of >50 mg/L (Figure 10). The organic carbon concentration is important in testing systems that use oxidizing agents as active substances. DOC concentrations are usually below 5 mg/L in low salinity test water but no DOC additions are made because the high POC values (>10 mg/L) in the NIOZ test water are considered to compensate for that. In other words, the total organic load in low salinity test water is sufficiently high.

An overview of the required water quality, with respect to the composition of total suspended solids, particulate organic carbon and dissolved organic carbon is given in Table 1.

Table 1. Requirements of salinity, TSS, POC and DOC in the test water for the high salinity and intermediate salinity and freshwater test regimes according to the G8-guidelines.

	High salinity	Intermediate salinity	Freshwater	units
Salinity	> 32*	3 – 32*	<3	PSU
Total Suspended Solids (TSS)	> 1	> 50**	> 50**	mg/L
Particulate Organic Carbon (POC)	> 1	> 5**	> 5**	mg/L
Dissolved Organic Carbon (DOC)	> 1	> 5	> 5	mg/L

*to obtain a 10 unit salinity difference either brine or fresh water may be added

**natural mud is added to increase TSS as needed, this also increases POC

4.2 Biological quality

In order to establish the biological efficacy of the BWTS it should be tested with water containing a high concentration of organisms as well as a sufficient biodiversity (§ 2.3.20 of G8). This is required by G8 to guarantee the effectiveness of the BWTS in different ecosystems across the globe. The variety of organisms in the influent test water should be documented according to the size classes mentioned in Table 2.

Natural water, originating from the coastal zone of the North Sea (high tide) and the inner Western Wadden Sea (low tide) will be used. The test period will cover the whole spring and early summer of the plankton growth season and therefore includes the natural occurring biodiversity and species succession. The ambient plankton content in terms of species diversity in the relevant size classes is very high. For instance in 2011 16 phyla and more than 70 species were detected during the test season (Table 3). Only 5 species and 3 phyla are required (§ 2.3.20 of G8).

Table 2. Minimal numbers and species diversity required at intake for different size classes and groups of organisms. 1 µm = 1 micron = 0.001 mm.

Intake test water		
Organism	unit	Variety
≥50 µm	> 10^5/ m^3	at least 5 species from at least 3 different phyla/divisions
≥10 and <50 µm	> 10^3/ mL	at least 5 species from at least 3 different phyla/divisions
heterotrophic bacteria	> 10^4/ mL	not further defined

The natural waters of the test area include a large range of organisms varying in sensitivity to mechanical stress, UV radiation or various active substances. Besides fragile organisms also plankton that is highly adapted to harsh environmental conditions, mostly hard shell organisms, are present in the test water.

For completeness, the plankton fraction <10 µm is also included in the NIOZ analyses although this is not required by the G8-guideline.

Table 3. Biodiversity as number of species in NIOZ test water according to phylum and to size class based on data from the 2011 spring and early summer test season. The test water contains at least 18 phyla with a total of 16 phyla in the 10-50 and >50 µm size classes. The total number of species in each G8-size class is 42 (8 phyla) for the 10≤µm<50, and 31 (10 phyla) for the ≥50µm size classes. Organisms <10µm that are not bacteria are not part of the D-2 regulation. The high test water biodiversity largely exceeds the G8-guideline (§2.3.20).

Phylum[a]	Number <10 µm	Number 10-50 µm	Number >50 µm
Amoebozoa		1	
Annelida			5
Arthropoda			13
Cercozoa		1	
Chlorophyta[b]	1	1	
Choanozoa	1		
Ciliophora		3	
Cnidaria			2
Cryptophyta	1		
Ctenophora			1
Echinodermata			1
Euglenozoa		1	
Haptophyta	3		
Mollusca			4
Myzozoa	2	6	1
Nematoda			1
Ochrophyta	12	28	2
Rotifera			1
Unknown[c]	4	1	
Total	**24**	**42**	**31**

[a] The taxonomic system is as follows: Kingdom (Archea, Bacteria, Animalia, Chromista ("Algae"), Plantae) – Subkingdom – Infrakingdom – Phylum – Subphylum - Division – Class – Subclass –Superorder – Order – Family – Genus – Species

[b] Division (no phylum for this group)

[c] The phylum 'unknown' contains several species of unidentified phytoplankton flagellates

4.3 General sampling strategy

Samples are generally taken:

1) In the harbour to assess test water quality before the pump. Harbour water samples are analysed regularly from February onwards in order to monitor the spring plankton bloom.
2) Immediately before the treatment equipment from the main pipeline but after the ballast pump that is used to pump up the test water from the harbour (control, T0),
3) Immediately after treatment from the main pipeline (treated, T0) and
4) During discharge from the main pipeline, after the pump, after 5 days (control and treated, T5) holding time (§ 2.3.2 and 2.3.26 G8-guidelines) and after completing a second passage through the BWTS when this step forms part of the treatment prescribed by the vendor of the BWTS, i.e. in the case of the Cathelco BWTS (treated only).

During ballast water tests samples will be taken sequentially, covering the entire intake or discharge periods.

During the tests the following sample sizes will be used:

1) Untreated water (control, T_0 and T_5): Sampling is conducted in-line, three times, with sample volumes of 20 L (>50 µm), 10 L and 2x 1 L. The 10 L samples are used to subsample for abiotic variables. The 1 L samples are used (1) to subsample for phytoplankton, bacteria, as well as for phytoplankton <10 µm and (2) microzooplankton (10≤µm<50). An additional 10 L sample is taken for an incubation experiment (T_0 only).
2) Treated water (intake, T_0): Sampling is conducted in-line, three times, with sample volumes of 1 m^3 (>50 µm) using 3 IBCs of 1000 L each. Furthermore, in-line sampling is conducted three times with a sample volume of 10 L and 2x 1 L. The 10 L samples are used to subsample for abiotic variables. The 1 L samples are used (1) to subsample for phytoplankton, bacteria, as well as for phytoplankton <10 µm and (2) microzooplankton (10≤µm<50). Additional samples of 10 L are taken for incubation experiments.
3) Treated (discharge, ≥T_5): Sampling is conducted in-line, three times, with sample volumes of 1 m^3 (>50 µm) using 3 IBCs of 1000 L each. Furthermore, in-line sampling is conducted three times with a sample volume of 10 L and 2x 1 L. The 10 L samples are used to subsample for abiotic variables. The 1 L samples are used (1) to subsample for phytoplankton, bacteria, as well as for phytoplankton <10 µm and (2) microzooplankton (10≤µm<50). Additional samples of 10 L each are taken for incubation experiments.

The (sub)-sample volumes taken from the in-line samples deviate from §2.3.32-33 (G8-guideline): 10≤µm<50: 1 L for untreated water and 10 L for treated water; bacteria: 0.5 L. Note that G8 only specifies sample sizes, but not which volume of these samples should actually be analysed. The reason for this deviation is that sample volumes of 10 L for organisms 10≤µm<50 and 0.5 L for heterotrophic bacteria are impractical: much smaller volumes will eventually be analysed without compromising the analytical accuracy. The sample volumes for pathogenic bacteria are 300 and 600 mL.

4.4 Abiotic variables: sampling and storing

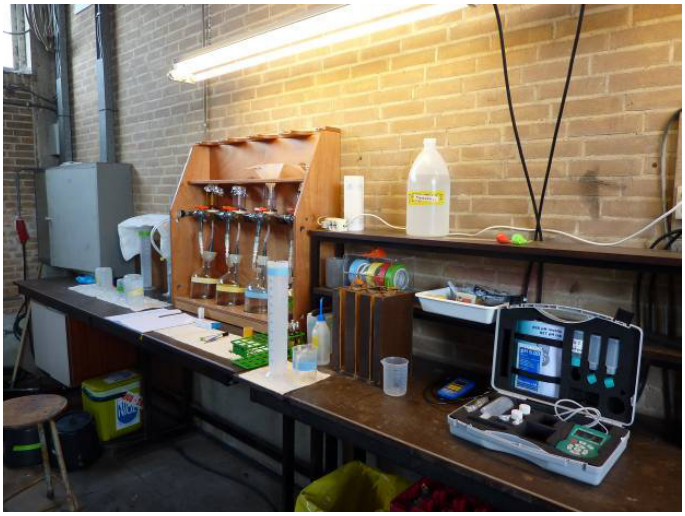

Figure 11. Navicula quay laboratory for first line handling of samples.

The measurement of the following abiotic (environmental) variables is required during sampling (G8 § 2.3.25):

1) salinity,
2) temperature,
3) Particulate Organic Carbon (POC),
4) Total Suspended Solids (TSS),
5) pH,
6) Dissolved Oxygen (DO) and
7) Dissolved Organic Carbon (DOC).

Turbidity is also mentioned in § 2.3.25 (G8) but experience has learned that it is difficult to reliably measure this variable in water samples of limited volume taken during intake or discharge. Furthermore, turbidity is a function of TSS, a variable that is measured accurately and with far better precision. In addition, NIOZ research has shown that at low TSS values a standard turbidity instrument underestimates the actual solid content of the water.

Rapid measurement and processing of samples for the basic variables is ensured by the NIOZ harbour laboratory on the Navicula and Pelagia quays of the test facility (Figure 11). A number of measurements is performed after sampling (e.g. temperature, salinity and pH), while the other samples (DOC, POC, TSS, DO) is processed for later analysis.

DOC samples are filtered through GF/C filters and sealed in pre-combusted glass ampoules or special screw cap bottles after adding hydrochloric acid (HCl). Sealed ampoules or bottles are stored at 4°C until analysis.

For TSS/POC measurements pre-weighed glass-fibre filters (GF/C) are used. Each filter is coded and stored individually in a Petri dish. The filtered water volume is not constant but depends on the particle concentration of the test water. The higher the particle concentration in the sample, the smaller the volume that can be filtered before clogging. Practical volumes are 100-1000 mL per sample. After filtration the filter is rinsed with demineralised water to remove sea salts. The filter is then put back in its original Petri dish and stored temporarily in a freezer at –20°C or long-term at -80°C until further analysis.

Glass Winkler bottles for oxygen (DO) are flushed at least three times their volume (ca. 120 mL) with test or control water. Great care is taken to avoid gas bubbles on the wall of the bottle or excessive mixing of air and water. Next, $MnCl_2$ and NaOH solutions are added to the bottle just below the surface by using dispenser units to fix the oxygen concentration. A stopper secured with a rubber band is put on the bottle and the bottle is gently mixed. Bottles are stored in a dark container filled with water of the same temperature as the samples until further analysis in the laboratory (Figure 12).

Figure 12. Glass bottles for Dissolved Oxygen (DO) measurements are stored submerged prior to analysis.

In special cases gastight tubing which is fitted to the sampling tubing is used to avoid exchange of gasses, such as CO_2 or oxygen, between water and surrounding atmosphere (Figure 13).

All environmental variables are measured in triplicate samples (§ 2.3.29 G8-guidelines).

From 2012 onwards, additional continuous measurements of temperature, salinity, flow rate, system pressure and turbidity will be made at intake and discharge by sampling via a specific sample port equipped with the appropriate monitoring instrumentation (e.g. S and T in Figure 3).

The data will be logged electronically and will be used to monitor basic system variables during intake and discharge of the BWTS tests.

Figure 13. Sampling point on a tank at the Navicula quay for sampling dissolved gasses such as dissolved oxygen.

4.5 Biological variables: sampling and storing

Biological variables that need to be considered (Regulation D-2 and § 2.3.20-21 G8-guidelines) are:

(1) Concentration and diversity of organisms ≥50 µm,
(2) Concentration and diversity of organisms 10≤µm<50,
(3) Concentration of heterotrophic bacteria,
(4) Concentration of coliform bacteria,
(5) Concentration of *Enterococcus* bacteria, and
(6) Concentration of *Vibrio cholera*.

The groups of organisms ≥50 µm and 10≤µm<50 consist of both algae (phytoplankton, mainly < 50 µm) and animals (zooplankton, mainly >50 µm). According to the Ballast Water Performance Standard (Regulation D-2 and § 4.7 G8-guidelines) only viable organisms must be counted at the end of the test in both the treatment and the control tanks. For both of the IMO relevant size classes (organisms ≥50 µm and 10≤µm<50), multiple methods of enumeration and of assessing the viability are applied at NIOZ to verify the results at a high level of confidence. In principle all methods should give a conclusive answer with respect to numbers and/or viability of the (remaining) organisms.

At NIOZ the viability of all organisms is not only measured at discharge but during intake as well. Furthermore, the concentration of phytoplankton <10 µm is measured. All biological variables are measured in triplicate samples (§ 2.3.29 G8-guidelines).

4.5.1 Sampling organisms ≥50 µm

The samples of the untreated water (20 L) are taken in triplicate, collected in clean buckets that are filled directly for volumetric measurements, and poured through a 50 µm sieve made from the same gauze as the sampling nets used for the 1 m^3 IBC samples.

Discharge water samples are collected using Hydrobios™ 50 µm diagonal mesh size nets, as recommended in MEPC 54/Inf.3, that are fitted into 1m^3 IBC's (Figure 14). Sampling is conducted via flexible hoses which are connected to the sampling points. To sample treated water the hose is put into the Hydrobios™ net. The whole sampling procedure will be timed in a way to cover the whole period of filling the ballast water tank with ca. 250 m^3 water. For practical reasons all organisms quantitatively retained on the 50 µm net are considered as larger than 50 µm in minimum dimension.

The organisms retained in the cod-end of the Hydrobios™ net are flushed into a beaker using a squeeze bottle containing filtered seawater. Organisms are kept in approximately 100 to 200 mL of filtered (≤0.2 µm filter) sterile water of the relevant salinity. Samples are transferred to the lab directly after sampling, treated for 2 hours with the viability stain 'Neutral Red', and counted. These samples are not stored.

Figure 14. Intermediate Bulk Containers (IBCs) of 1 m3 each. A 50 µm Hydrobios™ plankton net is fitted in the containers through the lid for easy sampling.

4.5.2 Sampling and storage of 10≤µm<50 organisms

Samples for the 10≤µm<50 fraction are taken as undisturbed (unfiltered) one litre samples. This size-fraction is not separated from the organisms <10 µm or >50 µm at sampling or during sample processing but during data analysis. This approach reduces damage to more delicate organisms as ciliates and non-armoured flagellates.

Whole, intact samples of 1 L are stored in the dark prior to analysis in the laboratory. The total concentration of 10≤µm<50 phytoplankton cells is measured in non-fixed samples. The concentration of dead phytoplankton cells is measured using SYTOX-Green, also in non-fixed samples. The difference between the concentrations of total and dead organisms is the concentration of viable organisms.

As a reserve a 5 mL sample is fixed with formaldehyde and stored at -80°C for up to one year. This fixed sample can no longer be used to determine the viability of the organisms.
The samples for 10≤µm<50 microzooplankton are fixed with Lugol's solution; they can be stored in a refrigerator for up to one year before counting takes place. The viability of the organisms is assessed from their intact morphology.

4.5.3 Sampling and storage of total and viable heterotrophic bacteria

To determine the total concentration of bacteria samples fixed with formaldehyde are frozen and stored at -80 °C until counts are made after staining with PicoGreen. Dead bacteria in non-fixed samples are measured in fresh test water using SYTOX-Green; these samples cannot be stored. The difference between the concentrations of total and dead bacteria is the concentration of viable bacteria.

4.5.4 Sampling of human pathogens

Live samples for microbiological analysis are taken in sterile bottles of 300 or 600 mL and sent to a special laboratory (Eurofins/ C.mark) for further analysis. The samples are transported immediately after sampling using a cooled transport container (4 °C).

4.5.5 Sampling for additional incubation (viability) experiments

Additional incubation experiments, which are not required by G8, serve to better assess the effect of the different ballast water treatment systems and the five day tank storage on cell viability and concentration. In case the treatment is insufficient and residual viable organisms remain present or resting stages or cysts germinate, growth of the plankton will be stimulated under favourable conditions. This incubation method also allows studies on the effect of the treatment over a period longer than 5 days (up to 20 days).

The experiments usually involve control and treated water that were sampled at T5, the day of discharge. They are performed in 10 L flasks in a climate room under optimal growth conditions for the plankton community, including irradiance, temperature and turbulence. A sufficiently high level of nutrients is ensured through the addition of nitrate, phosphate and silicate, favouring phytoplankton growth and stimulating cyst germination throughout these experiments. The standard variables measured include phytoplankton and microzooplankton abundance (<10µm and 10≤µm<50), the viability of phytoplankton (PAM fluorimetry) and the abundance of bacteria. Usually the incubation time is seven days. In more elaborate experiments samples are taken daily and may include multiple analyses of phytoplankton and heterotrophic bacteria.

5 MEASUREMENT OF VARIABLES

A list of all abiotic and biotic variables that are required by G8 is provided in Table 5.1. All variables are measured in samples of test water, taken during intake and discharge as required. Turbidity is a function of TSS, and because turbidity is difficult to measure reliably at relatively low TSS concentrations, this variable is only measured in-line and not in separate samples. *Vibrio cholerae* is not present in NIOZ test water, cannot be added, and hence is not sampled for. An additional measurement is that of phytoplankton <10 µm, which is not an IMO requirement (Table 5.1).

All measurements are described in the Standard Operating Procedures (SOPs) that are listed in section 3 of this project plan. Samples for E. coli and enterococci are outsourced and analysed according to NEN-ISO standards (Table 5.1). A brief description of all relevant methods is given in the following paragraphs.

5.1 Abiotic variables

5.1.1 Salinity, Temperature and pH

Water samples for salinity, temperature and pH are collected in 10 L buckets. Measurements are either done immediately or after storage (maximum 6 hours) in the dark and at ambient temperature. Salinity is measured with a digital conductivity meter. Temperature is measured with a calibrated digital thermometer. pH is measured with a calibrated digital pH meter.

5.1.2 TSS/POC (Total Suspended Solids/Particulate Organic Carbon)

For TSS analysis filters are dried at 60°C for at least 8 hours and weighed again. The concentration of TSS per litre can be calculated from the sample volume and the weight difference of the filter before and after sampling. TSS is expressed as mg/L.

Next, to determine the POC concentration the same filter is combusted overnight at 500°C and allowed to cool in a dessicator and weighed again. The POC is calculated from the weight difference between this measurement and the dry TSS weight. POC is expressed as mg C/L.

5.1.3 Dissolved Oxygen (DO)

Fixed samples in Winkler bottles are acidified with H_2SO_4 prior to measuring the optical density (OD) at 456 nm with a spectrophotometer. The oxygen concentration is calculated using standards and expressed as µM O_2/L (or mg O_2/L = µM O_2 * 0.032). Since both salinity and temperature change over the season the oxygen concentrations is expressed as percentage relative to the natural saturation value for the given temperature and salinity.

5.1.4 Dissolved Organic Carbon (DOC)

The DOC concentration is determined in the laboratory by a high temperature combustion method using a Shimadzu TOC-Vcpn analyser according to Reinthaler & Herndl (2005). Standards are prepared with potassium hydrogen phthalate (Nacalao Tesque, Inc, Kioto, Japan). The mean concentration of triplicate injections of each sample (three in total) is calculated. The average analytical precision of the instrument is <3 %.

Table 4. List of variables measured in land-based tests at NIOZ

Variable	unit	IMO required	Reference
Salinity	PSU	Y	SOP
Temperature	°C	Y	SOP
pH	-	Y	SOP
TSS	mg/L	Y	SOP
Particulate Organic Carbon	mg/L	Y	SOP
Dissolved Oxygen	% saturation	Y	SOP
Dissolved Organic Carbon	mg/L	Y	SOP
Viable organisms ≥50 µm, including diversity	number per m^3, number of phyla and species	Y	SOP
Organisms 10-50 µm (phytoplankton)	number per mL	Y	SOP
Phytoplankton diversity	number of phyla and species	Y	SOP
Phytoplankton viability (PAM fluorimetry)	Fv/Fm	Y	SOP
Phytoplankton viability (SYTOX Green)	number per mL	Y	SOP
Organisms 10-50 µm (microzooplankton)	number per mL	Y	SOP
Microzooplankton diversity	number of phyla and species	Y	SOP
Microzooplankton viability	+ or -	Y	SOP
Organisms <10 µm (phytoplankton)	number per mL	N	SOP
Heterotrophic bacteria	number per mL	Y	SOP
E. coli	cfu per 100 mL	Y	NEN-EN-ISO 9308-1
Enterococci	cfu per 100 mL	Y	NEN-EN-ISO 7899-2

5.2 Biological variables

5.2.1 Counting of organisms ≥50 µm

For minimum dimension measurements the "body" of the organism should be measured, i.e. not antennae, tails etc. Examples are presented in Figure 15.

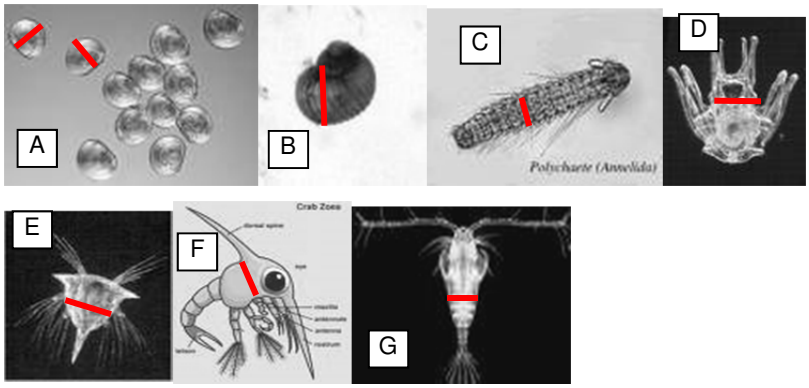

Figure 15. Minimum dimension measurements (red line) in selected organism types: A = bivalve larvae, B = gastropod larvae, C = worm, D = echinodermata larvae, E and F = crustacean larvae and G = copepod.

The viability of the organisms is assessed with Neutral Red, which stains living organisms only and does not affect their survival rate. This viability assessment remains unaffected by the possible

death of organisms during staining or during sample analysis due to, for instance, warming of the sample. This is because organisms that die after addition of the Neutral Red will still be clearly stained, while those already dead prior to the addition will not be stained.

Neutral Red is pipetted in a ratio that yields an end concentration of approx. 1:50.000. The Neutral Red stock solution is 1:2.000, i.e. approximately 4 mL of stock solution is needed to stain a sample of 100 mL. The staining time is ≥ 2 hours. Stained samples are filtered over a 30 µm sieve and flushed into a Bogorov-dish with filtered seawater. Samples are analysed using a binocular with a 20x magnification for counting and up to 80x for species identification and measurements when necessary.

Neutral Red stains all major plankton groups, including phytoplankton, but it seems to have some limitations for bivalve larvae. For the latter movement, including that of hart and gill is used to verify viability. This is dependent on the expertise of the person analysing the samples. Therefore, only persons with a dedicated training period will analyse samples. Organisms that are able to swim are also considered alive. In doubt, the organism can be poked with a dissection needle. The procedure is outlined in Figure 16.

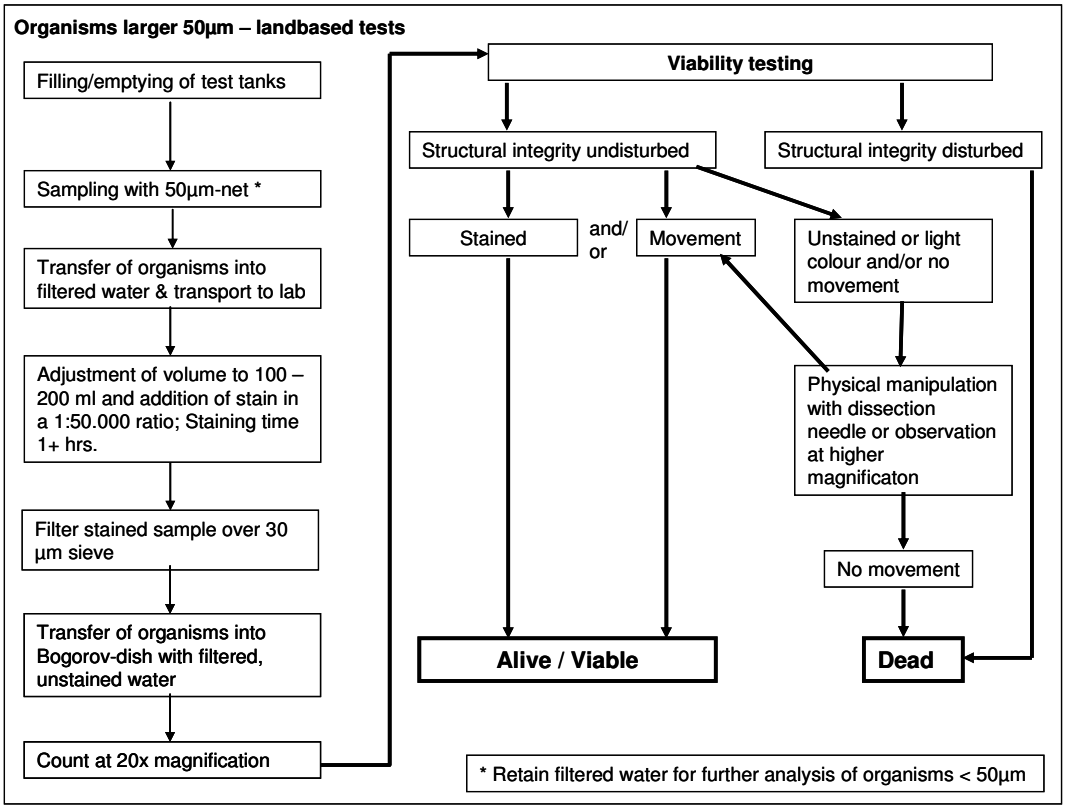

Figure 16. Sampling and viability assessment for organisms larger than 50 µm during land-based tests.

5.2.2 Counting of organisms 10≤µm<50

5.2.2.1 Counting viable microzooplankton

The sample bottles are transferred to the laboratory and left undisturbed in the dark for all organisms and material to settle. After at least 24 hours the full bottle weight is measured. Using a water-jet pump and specialized tubing, the supernatant is aspirated. The bottle is weighed once more. The concentrate is transferred to small storage bottles that can be stored refrigerated for one year. The bottle is weighed once more to determine the sample and concentrate volumes.

The samples are analyzed with an inverted microscope at 200x magnification (method by Utermöhl). The fixed samples (or sub-samples thereof) are transferred into settling chambers and

neutralized using sodium thiosulfate. After this, the sample is stained using Bengal rose stain. This stain specifically stains organic material and helps to identify organisms between sediment particles. After staining the samples are left undisturbed in the dark to settle. The time of settling depends on the settling chamber used, but should be at least 1 hour per cm height of the settling chamber. The iodide component of the Lugol's solution increases the weight of particles. Therefore they settle on the bottom-glass and can be counted. Live-dead-separation in these samples is mainly based on the structural integrity of organisms. This method can be applied for both zoo- and phytoplankton. Nevertheless certain groups are known to be affected by this standard method of fixation and therefore they will be systematically underestimated, if present.

5.2.2.2. Counting viable phytoplankton

Organisms in the 10≤µm<50 size class will be analyzed via flow cytometry (Figure 17), a semi-automated method used in the NIOZ for the counting of phytoplankton, bacteria and viruses. In principle, flow cytometry allows to assess a complete view of the effectiveness of the applied treatment technique for all organisms, irrespective of their size (Veldhuis & Kraay, 2000). By applying special staining techniques the numerical abundance of heterotrophic as well as autotrophic bacteria can also be estimated. The vitality of the different organisms present will be addressed by using a specific fluorescent dye method (Veldhuis et al. 2001, Cassoti et al. 2005, Veldhuis et al. 2006, Peperzak & Brussaard 2011). In addition, the viability will be assessed by incubating discharge water (see § 4.5.5). The diversity of the phytoplankton is measured by microscopy of Lugol-fixed samples of the NIOZ test water by Koeman & Bijkerk (The Netherlands). This company is actively involved in international quality assessments (http://www.planktonforum.eu/)

Three replicate samples from both control and treated water are placed in the carousel of a bench top flow cytometer (Beckman Coulter XL-MCL or Becton Dickson Canto II), of which the Canto flow cytometer has an elaborate quality control system. All procedures and handling are conducted according to standard procedures (e.g. Shapiro 2003).

Samples will be counted using standard protocols covering the particles in the size range of ca. 2 to 50 µm. Total analysis time will be equal to an exact sampling volume of 1 mL or otherwise when relevant. Of all particles present in the volume counted, the cell size and the presence or absence of chlorophyll-*a* fluorescence will be measured. Only phytoplankton has chlorophyll-*a* fluorescence (Figure 18a,b).

Absolute numbers, cell sizes and chlorophyll-*a* content of the particles will be analyzed using the software package FCS Express V3 or V4 (DeNovo, US). Cell sizes will be estimated relative to 10µm standard fluorescent beads (Flow-Check Fluorospheres, Beckman Coulter #660539) or relative to 10 and 50 µm beads on the Canto II.

For measuring viable phytoplankton, three subsamples will be stained with SYTOX Green (Veldhuis et al, 2001). This nucleic acid specific dye only stains DNA of cells with a compromised cell membrane. Of each phytoplankton cell present the green SYTOX fluorescence (Figure 15b) will be determined and compared with the green autofluorescent signal (Veldhuis et al, 2001, Cassoti et al, 2005, Peperzak & Brussaard 2011).

Figure 17. Bench top flow cytometer (Becton Dickinson Canto II), an instrument to enumerate live and dead organisms <50 µm.

Figure 18. (a) Epifluorescence microscopic picture of a live phytoplankton cell. The red signal is due to the presence of chlorophyll-*a*, and (b) a dead phytoplankton cell with a yellow-green fluorescence of the nucleus after staining with SYTOX Green.

5.2.3 Counting total heterotrophic bacteria

The classical method for counting bacteria in many applications is based on plating on selective media. Unfortunately, for studies in the aquatic environment this approach is by far insufficient for various reasons (Gasol & Del Giorgio 2000). Therefore, the total bacteria concentration in fixed samples is accurately determined by flow cytometry using the DNA-specific stain PicoGreen (cf. Gasol & Del Giorgio 2000, Veldhuis et al. 1997).

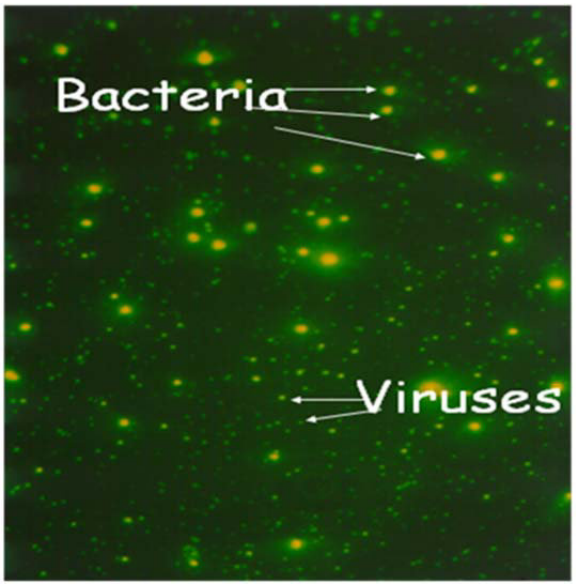

Figure 19. Epifluorescence microscopic picture showing bacteria and viruses in a natural water sample. The yellow-green fluorescence signal is DNA stained with a nucleic acid dye.

The dye PicoGreen is a green nucleic acid specific dye that only stains dsDNA, with little or no cross-over for ssDNA and RNA (Veldhuis et al, 1997). This makes the staining method ideal to for staining of DNA and therefore to determine bacterial abundance. Flow cytometric analysis shows a clear signal with an excellent signal to noise ratio and bacteria are made visible easily and distinguishable from viruses and larger organisms. This approach has extensively been compared with bacteria staining and counting using an epifluorescent microscope (Figure 19), resulting in nearly identical results. However, because the flow cytometer method is much faster (results are obtained within 100 seconds and over 100 samples can be analyzed per day), and highly reproducible this counting method is to be preferred above the far more time consuming and labour intensive microscopic observations.

5.2.4 Human pathogens

The samples for microbiological analysis are taken in special bottles of 300 or 600 mL and send to a special laboratory (Eurofins/ C-mark, Quality system: Testing RVA L154) for further analysis. All analyses are carried out according to NEN-ISO standards.

Escherichia coli

Analysis for Escherichia coli is carried out according to NEN-EN-ISO 9308-1 for the analysis of surface waters. For this the samples are filtered through membrane filters (pore size 0.45 µm) and these filters are incubated on a selective agar plate. Incubation is 4.5 ± 0.5 hours at 37 ± 1°C and then another 19.5 ± 0.5 hours at 44 ± 0.5 °C. After that the incubated filters are transferred on sterile filters soaked with Indol reagent. For colonies of E. coli this yields a red colour. These red colonies are counted and set into relation to the sample volume. Results are confirmed via a positive and a negative control. For the latter sterilized water is incubated like a regular sample and to confirm the results it may only yield less than 1 colony forming unit (cfu) per mL. The positive control uses a special strain of E. coli also incubated like a normal sample to confirm that this species can grow and form colonies on the used media.

Enterococci group

Analysis for this group is carried out according NEN-EN-ISO 7899-2. For this the samples are filtered through membrane filters (pore size 0.45 µm) and these filters are incubated on a selective agar plate. Incubation is 44 ± 4 hours at 36 ± 2°C on Slanetz & Bartley medium. After that red and pink colonies are counted. If the presence of enterococcus bacteria can be suspected after the colour of the colonies the filter is transferred to a pre-heated, selective agar plate and incubated for another 2 hours at 44 ± 0,5 °C. After that the medium is examined whether or not a brown to black colour can be found in it. Results are confirmed via a positive and a negative control. For the latter sterilized water is incubated like a regular sample and to confirm the results it may only yield less than 1 colony forming unit (cfu) per 100 mL. The positive control uses a strain of *Enterococcus faecium*.

5.2.5.2 PAM measurement for total phytoplankton viability

The photochemical efficiency of photosystem II is an indicator of the physiological 'health' condition of phytoplankton cells. It is a bulk variable that is measured using a Pulse Amplitude Modulated (PAM) fluorimeter (Schreiber et al 1993, Figure 20). The simple fluorescence ratio Fv/Fm gives a qualitative indication of the photosynthetic efficiency of the phytoplankton community. In addition, the maximum fluorescence value Fm is an indication of phytoplankton biomass.

Prior to the measurement the sample is kept in the dark for at least 30 minutes. 3 mL of unfiltered sample water (control and treated, each in triplicate) are filled into a glass cuvette and analysed within 2 minutes. In the case of a high photosynthetic efficiency of the bulk phytoplankton community, samples can be filtered using 50 and 10 µm Hydrobios™ gauze to determine the exact size class of the viable phytoplankton fraction.

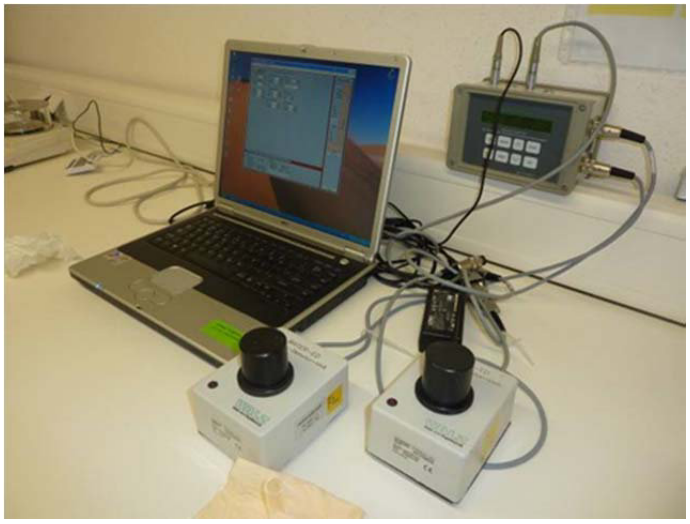

Figure 20. PAM fluorimetry, a fast method to determine (bulk) phytoplankton biomass and the physiological condition of the photosynthetic apparatus of the cells.

5.2.5.3 Counting phytoplankton <10 µm

Organisms in this size class will be analyzed via flow cytometry, as explained for the 10≤µm<50 phytoplankton size class.

6 QUALITY ASSURANCE/ QUALITY CONTROL (QA/ QC)

The Quality Management Program (QMP) addresses the quality control management structure and policies of the test facility.

Sampling and analysis standard operating protocols (SOPs) contain QA/QC measures where applicable.

6.1 Ballast water tests

For all ballast water test scenarios piping and instrumentation diagrams are available. Prior to each test a tool box meeting is held to ensure that the proper procedures are followed during intake and discharge. During the ballast water project all samples are taken, stored and analysed according to the dedicated SOPs. Prior to all tests sample codes are assigned following the rules explained in Table 4.

Because the test site is within very short distance of the main NIOZ building all samples containing fresh and live material are immediately transported to the laboratory for direct analysis. The sample storage flasks as well as cryovial boxes are labelled with the same coloured labels and codes. Samples that are fixed for long-term storage are stored in specifically designated refrigerators (4°C) and freezers (-20°C, -80°C).

The samples for microbiological analysis of the presence and number of human pathogens will be taken in special bottles of 300 and 600 mL and sent to "Eurofins/C.mark" in Heerenveen (accreditation certificate: RvA lab. no. L043). The samples will be transported immediately after sampling using a cooled transport container (4 °C).

6.2 Laboratory analyses

The analyses of abiotic and biological variables are described in general in other parts of this project plan. Detailed descriptions of each analysis are available. These Standard Operating Procedures (SOPs) of sampling, sample storage, sample analyses, data analyses and data management are part of the NIOZ Ballast Water QMP. Specific quality assurance and quality control measures are contained in each SOP.

6.3 Data analysis

The sample codes assigned a priori to the harbour tests (Table 4) are also used in data handling, i.e. the transfer of data from laboratory instruments to Excel™ files, dedicated to specific analyses. All data files are collected on a NIOZ network disk that is backed-up at least once a day. The separate data files are combined in one Excel™ file in which all appropriate calculations for D-2 and G8 will be conducted. The data on the NIOZ network disk are accessible to authorised NIOZ test facility personnel only.

Statistical analyses will be performed in Excel™ version 14. Additional analyses will be performed in either SYSTAT version 13 or Primer version 6. SYSTAT and Primer allow for more sophisticated statistical analyses of the BWTS' performance than the t-tests that are recommended in §2.3.37 of the G8-guidelines. The scientific hypothesis that will be tested, the so-called null-hypothesis, is that there are no differences between treated and control water samples.

Quality data are compiled in tables, and when possible visualised in diagrams such as, for instance, Shewhart control charts.

NIOZ will report the total number of tests that were needed to meet the D2-standard five times for each salinity range.

Table 5. Example of the assignment of coloured codes prior to tests. Sample flasks contain the appropriate code on a coloured label to prevent misidentification. T0 is the day of treatment and Tx is the x^{th} day of sampling. Usually x = 5 at discharge. Test numbers are Roman numerals (I, II, III, etc.) using a new number for every test (e.g. I to X). Replicates are denoted with normal numbers (1, 2, 3, etc.).

The example codes I-T5-M(1-4)-1 is, for each manufacturer, the first replicate sample of Test I on day 5 (T5).

Sample	Quay	Code	Label colour
Wadden Sea (harbour inlet)	Navicula	WSn	Red
Wadden Sea (harbour inlet)	Pelagia	WSp	Red+Orange
Control tank (T0)	Navicula	Cn	Green
Control tank (T0)	Pelagia	Cp	Green+Red
Treatment tanks for T0 to Tx:		e.g.:	
Manufacturer 1	Navicula	I-T5-M1-1	Orange
Manufacturer 2	Navicula	I-T5-M2-1	Yellow
Manufacturer 3	Pelagia	I-T5-M3-1	Purple
Manufacturer 4	Pelagia	I-T5-M4-1	Blue

7 REFERENCES

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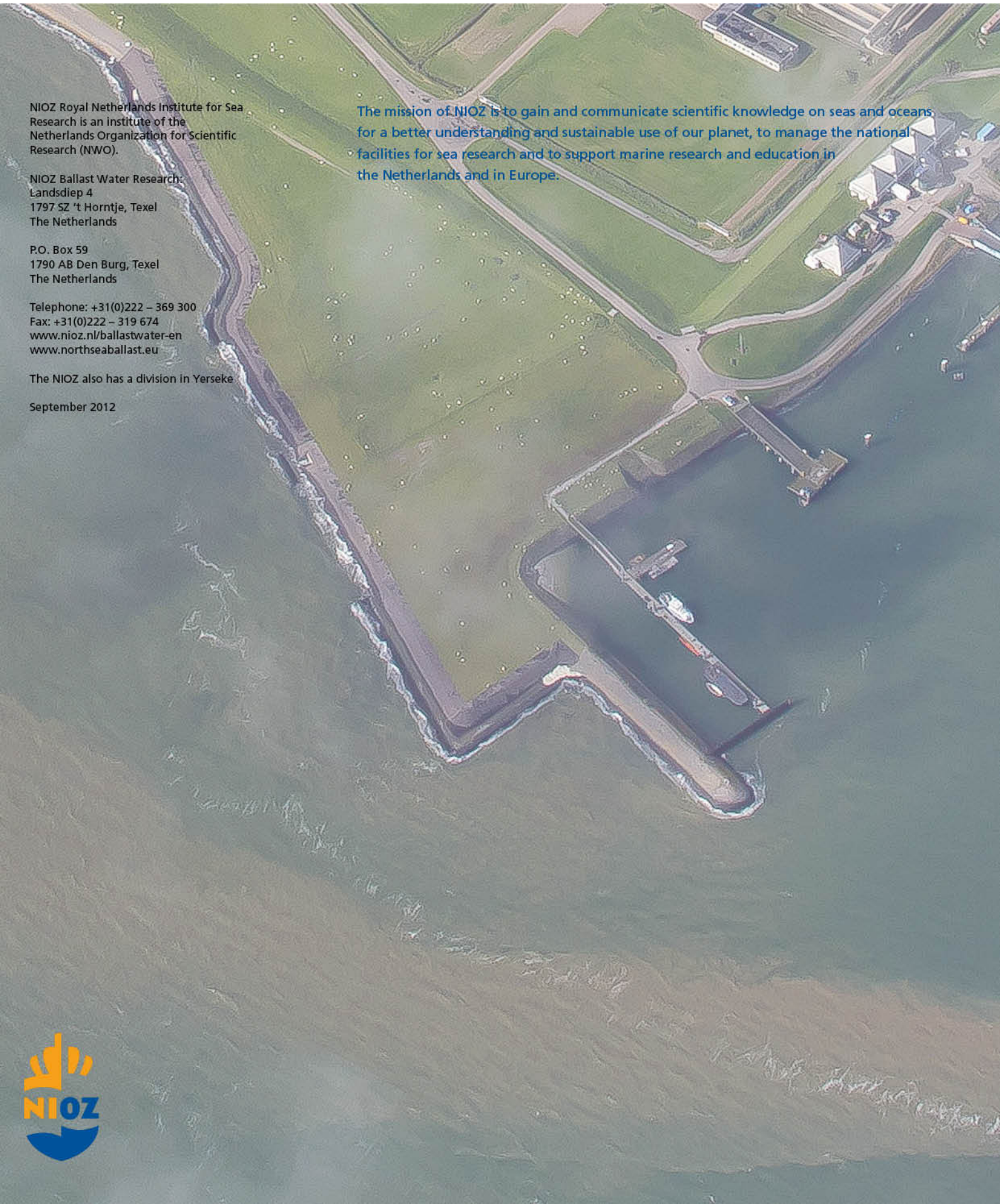

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September 2012

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Revision	Date	Description	Author	Checked	Approved
02	07/10/13	Renumbered	PH	SRE	RF
01	30/09/13	Initial Issue	PH	SRE	RF
00	20/09/13	Initial Issue	PH	SRE	RF